BEST AVAILABLE COPY

APPENDIX A

Vol. 177, No. 6

A Mutant Phosphoenolpyruvate Carboxykinase in *Escherichia coli* Conferring Oxaloacetate Decarboxylase Activity

SHAO-YI HOU, YUN-PENG CHAO, AND JAMES C. LIAO*

Department of Chemical Engineering, Texas A&M University, College Station, Texas 77843-3122

The phosphoenolpyruvate carboxykinase in *Escherichia coli* (encoded by *pck*) catalyzes the conversion from oxaloacetate (OAA) to phosphoenolpyruvate under gluconeogenic conditions. We report here the characterization of two mutant alleles, *pck-51* and *pck-53*, both of which are point mutations leading to single amino acid changes (D to N at position 268 and G to S at position 284, respectively). Pck51 is an altered-activity mutant that catalyzes the conversion from OAA to pyruvate (OAA decarboxylase activity). This new activity was not detected from the wild-type Pck, and it complements the *pck* null mutation only in a *pps*+ background. Pck53 is a reduced-activity mutant that complements the *pck* null mutation in a strain-dependent fashion.

The *Escherichia coli* phosphoenolpyruvate carboxykinase (Pck; also known as PEPCK in other organisms) is a gluconeogenic enzyme that converts oxaloacetate (OAA) to phosphoenolpyruvate (PEP) at the expense of ATP. This enzyme, together with the NAD- and NADP-dependent malic enzymes, is important for *E. coli* growth on C_4 carbon sources such as succinate (see Fig. 1). Strains deficient in all three enzymes are unable to grow on C_4 carbon sources, whereas strains deficient in one of these three enzymes can still grow. However, a *pck pps* (encoding PEP synthase) double mutant does not grow on C_4 carbon sources because of the lack of PEP. The *pck* gene has been cloned, sequenced, and characterized (9, 12), and its transcription has been shown to be regulated by catabolite repression (8). Other ATP-dependent Pck genes in *Saccharomyces cerevisiae* (18), *Trypanosoma brucei* (15), and *Rhizobium* spp. (14a) have been identified and sequenced.

By overexpressing Pck, it was found that this enzyme is one of the key factors controlling growth rate when *E. coli* is grown on succinate (3). However, medium- to high-level overexpression of this enzyme causes severe growth inhibition (3), which is independent of the carbon source (unpublished results). This growth inhibition may be attributed to protein overproduction because of metabolic burden and extra ribosome load. To control for the protein overexpression effect, we isolated inactive Pck mutants that have similar expression levels. During this process, we obtained a mutant Pck that confers OAA decarboxylase (Oad) activity (converting OAA to pyruvate), which was not detected in the wild-type *E. coli*. This mutant will be useful in defining the physiological role of Pck and its active sites.

Mutant isolation was performed as described below. Plasmid pCK601 (2) containing the *pck* gene was transformed into a *mutD5* strain (4). The plasmid was then purified and retransformed into HG4 (*pck-2 pps-3 pyrD his tyrA*) (6) (from Hughes Goldie, University of Saskatchewan, Saskatoon, Canada). The transformants were screened for the inability to complement the *pck* mutation on the chromosome of HG4. To do so, these transformants were first plated on Luria-Bertani plates (13) and then replica plated on succinate M9 (13) plates with tyrosine, uracil, and histidine, which were required for HG4 to grow on minimal media. Growth on succinate indicates that

the strain contains a *pck*+ gene, most likely from the unmutated pCK601. Colonies that did not grow on the succinate M9 plate were picked as potential mutants in which the plasmid-borne *pck* had been mutated. These candidate colonies were grown in Luria-Bertani medium, and total cellular proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and examined for Pck overexpression. Most of these colonies did not show overexpressed Pck with the estimated molecular weight (51,000) (12). Two of the colonies, however, expressed similar amounts of protein at the same migration distance as the control strain that overexpressed the wild-type Pck. The plasmids in these two colonies were designated pCK601m1 and pCK601m3, and the genes were designated *pck-51* and *pck-53*, respectively. The Pck activity of these mutants was measured by detecting the production of OAA from PEP as described previously (7). Briefly, the cell extract was mixed with 1.8 ml of reaction buffer (containing 50 mM $NaHCO_3$, 80 mM $MgCl_2$, and 0.1 mM Tris, pH 7.5), 0.1 ml of 10 mM PEP, and 0.1 ml of 4 mM ADP. After incubation for 20, 40, or 60 min, the reaction was stopped by adding 0.75 ml of ethanol. OAA was then measured spectrophotometrically at A_{520} after the addition of fast violet B and 10 min of color development. No Pck activity was detected for these two mutants by this assay.

To probe the cause of growth inhibition observed when Pck is overexpressed (3), these two mutants were overexpressed and their effects on growth rate were examined. Pck51 did not show growth inhibition at high induction levels, but Pck53 did, even though both failed to show Pck activity when assayed by the colorimetric method. It is possible that the above-mentioned complementation test and the enzyme assay were not sensitive enough to detect reduced activities. Therefore, we used a different strain background for the complementation test, which was followed by an NADH-linked enzyme assay. To do so, we transformed pCK601m1, pCK601m3, and pCK601 into JCL1305 (*pck maeA maeB*) (from Elliot Juni, University of Michigan, Ann Arbor), which is deficient in Pck and both NAD- and NADP-dependent malic enzymes (10), as shown in Fig. 1. If any of these plasmids expressed Pck activity or Oad activity, the strain would grow on succinate. Unexpectedly, JCL1305 overexpressing either Pck50 or Pck53 grew on succinate, although the Pck53-overproducing strain grew more slowly than the wild-type Pck-overproducing strain (Fig. 2). As a control, JCL1305, containing the cloning vector (pJF118EH [5]), did not grow on succinate. The positive result of comple-

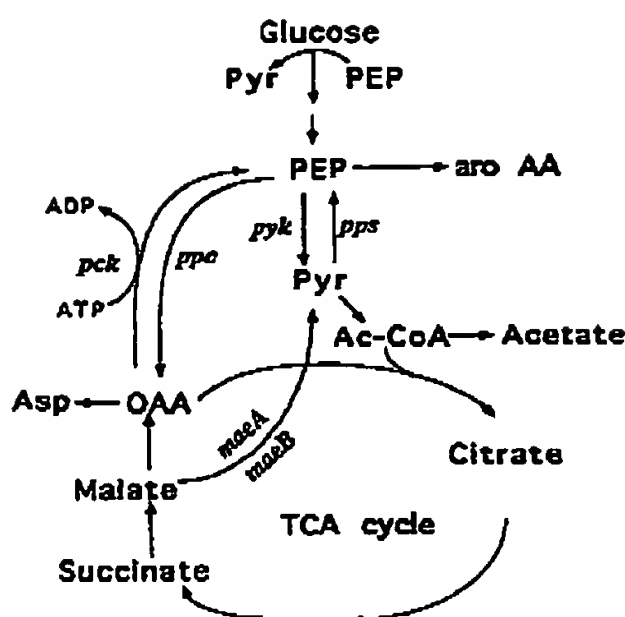* Corresponding author. Phone: (409) 845-9807. Fax: (409) 845-6446.

FIG. 1. Metabolic pathways related to Pck. Abbreviations: pps, PEP synthase; pyk, pyruvate kinase; pyc, PEP carboxylase; maeA, NAD-dependent malic enzyme; maeB, NADP-dependent malic enzyme; pyr, pyruvate; aro AA, aromatic amino acids; ASP, aspartate.
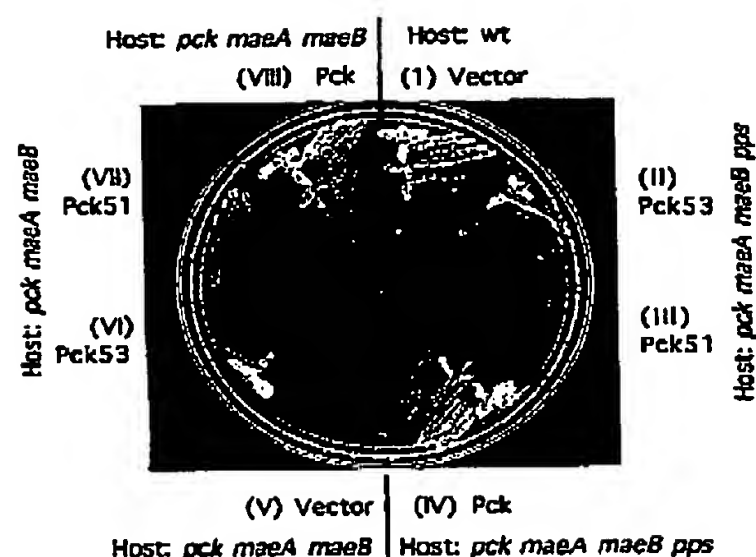

FIG. 2. Complementation test of Pck51 and Pck53. Strains harboring plasmids pCK601 (Pck), pCK601m1 (Pck51), pCK601m3 (Pck53), and pJF118EH (vector) were grown on the succinate M9 plate. The host strains in sectors V to VIII were JCL1305 pck maeA maeB. The host strains in sectors II to IV were JCL1441 (same as JCL1305 but pps). The host in sector I was a wild-type (wt) prototrophic E. coli containing pJF118EH as a positive control, while sector V was a negative control (JCL1305/pJF118EH).

mentation can be attributed to either the Pck or the Oad activity that has been demonstrated in yeast Pck (1) and chicken liver mitochondrial Pck (14). To distinguish between these two activities, we repeated the complementation test using JCL1441 (pck maeA maeB pps::Mu dII1734). This strain was constructed by P1 transduction of the pps::Mu dII1734 marker from JCL1362 (16) to JCL1305. If the above positive results were due to Oad activity, these Pck mutants would not support growth on succinate in this strain background. Indeed, Pck51 did not support growth of JCL1441 on succinate, while Pck53 and the wild-type Pck did (Fig. 2). These data suggest that Pck51 is an altered-activity mutant exhibiting Oad activity and that Pck53 is a reduced-activity mutant.

To confirm these results biochemically, we used an NADH-linked enzyme assay, which was modified from a published Oad assay, to detect both Pck activity and Oad activity (17). Briefly, cultures of JCL1305 harboring pCK601, pCK601m1, pCK601m3, or pJF118EH (the cloning vector) were harvested at early stationary phase, spun for 15 min at 12,000 × g, and resuspended in a buffer containing 0.1 M potassium phosphate (pH 8.0) and 4 mM MgCl$_2$. The cells were disrupted by passing them through a French pressure chamber at 800 lb/in^2. The cell extracts were used in the enzyme assay immediately. The assay mixture contained 0.1 M potassium phosphate (pH 8.0), 4 mM MgCl$_2$, 4 mM OAA, and 4 mM ATP (where indicated). Samples were taken at 0, 40, and 60 min and assayed for OAA and

pyruvate levels in the reaction mixture. OAA levels were determined in a solution containing 0.1 M potassium phosphate buffer pH 7.6 and 0.5 μM NADH by the decrease in A_{340} due to a stoichiometric decrease in NADH after the addition of malate dehydrogenase to this assay mixture (17). After A_{340} stabilized, lactate dehydrogenase was added, and the second decrease in A_{340} was used to quantitate the amount of pyruvate. Because lactate dehydrogenase contains significant amounts of malate dehydrogenase, it is imperative to measure OAA prior to measuring pyruvate. A control reaction with no cell extract was used to measure spontaneous decarboxylation of OAA to pyruvate. The rate of pyruvate production was reported as the Oad activity, and the rate of OAA disappearance minus the pyruvate production was taken as the Pck activity. Indeed, we found that Pck51 exhibited no Pck activity but contained significant Oad activity, while the wild-type Pck did not show significant Oad activity (Table 1). On the other hand, Pck53 showed reduced Pck activity but no Oad activity (Table 1). The Oad activity did not require but was activated by ATP, a characteristic consistent with the yeast Pck.

To determine the nature of mutation in pck-51 and pck-53, we first located the sites of mutation in both alleles to the SacI-ClaI fragment by DNA fragment complementation. We showed that all the phenotypes associated with Pck51 or Pck53 were conferred by this fragment. Therefore, the SacI-ClaI frag-

TABLE 1. Oad and Pck activities with or without ATP[a]

Plasmid	Without ATP		With ATP (4 mM)	
	Oad activity	Pck activity	Oad activity	Pck activity
pCK601 (pck$^+$)	0.01 ± 0.004	0.018 ± 0.009	0.014 ± 0.005	0.15 ± 0.015
pCK601m1 (pck-51)	0.064 ± 0.001	0.009 ± 0.003	0.100 ± 0.002	0.036 ± 0.005
pCK601m3 (pck-53)	0.009 ± 0.006	0.006 ± 0.008	0.028 ± 0.011	0.10 ± 0.016
pJF118EH (vector)	0.015 ± 0.01	0.006 ± 0.021	0.015 ± 0.01	0.020 ± 0.030

[a] The host strain was JCL1305 deficient in Pck and both NAD- and NADP-dependent malic enzymes. All values are in micromoles per milligram per minute.

Nucleotide sequence around the mutation sites:

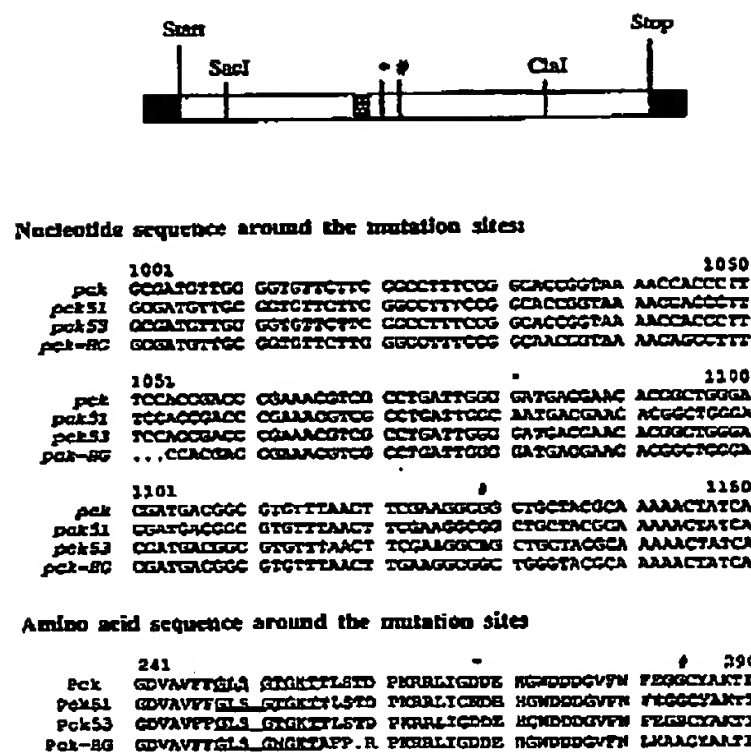Amino acid sequence around the mutation sites

FIG. 3. Nucleotide and the predicted amino acid sequences of Pck51 and Pck53 around the mutation sites. The top diagram shows the relative position of the sequenced region (SacI-ClaI) and the mutation sites for Pck51 (★) and Pck53 (●). The consensus ATP binding site is shown by the shaded region in the diagram and the underlined region in the amino acid sequence. Pck·HG indicates the sequence reported in the literature (12).

FIG. 4. A proposed mechanism of E. coli Pck- and Pck51-mediated reaction. Paths I and II lead to the formation of PEP and pyruvate, respectively. Wild-type Pck favors the former, while Pck51 favors the latter. R and E, ADP and the enzyme, respectively. The dashed arrow indicates stimulation of Oad activity by ATP.

ments from pck-51 and pck-53 were sequenced by use of SequiTherm cycle sequencing reagents (Epicentre Technologies, Madison, Wis.). DNA sequencing data (Fig. 3) show that the pck-51 allele is a point mutation from G to A at position 1081 leading to an amino acid change from Asp to Asn at position 268. Pck53 is also a point mutation from G to A at position 1129 leading to a change from Gly to Ser at position 284. Note that the numbers are based on the best sequence alignment with the published E. coli pck sequence (12). Although the sequence data from pck-51 and pck-53 agree with each other except at the mutation sites, they differ at some positions from the published sequence while keeping the reading frame largely unchanged. We therefore sequenced part of the SacI-ClaI region from the wild-type pck, and the results confirmed our sequence for pck-51 and pck-53. This discrepancy is perhaps attributable to a potential sequencing error in the published sequence (8a).

Oad activity was detected from the ATP-dependent Pck in S. cerevisiae and from the GTP-dependent Pck in chicken liver mitochondria but not from E. coli Pck. A sequential mechanism of Pck reaction has been suggested for the yeast Pck, which involves the Oad activity followed by a pyruvate kinase-like activity (1). A concerted mechanism has also been postulated for the liver Pck (19). The mechanism for E. coli Pck is still unknown but may involve a transitional enolpyruvate intermediate, which is stabilized by ATP. The nucleophilic enolpyruvate then attacks either the γ-phosphorus of ATP (Fig. 4, path I) or a proton (Fig. 4, path II) to yield PEP or pyruvate, respectively. The wild-type E. coli Pck favors the former, while Pck51 favors the latter, presumably because the mutation affects ATP binding to the enzyme. This hypothesis is supported
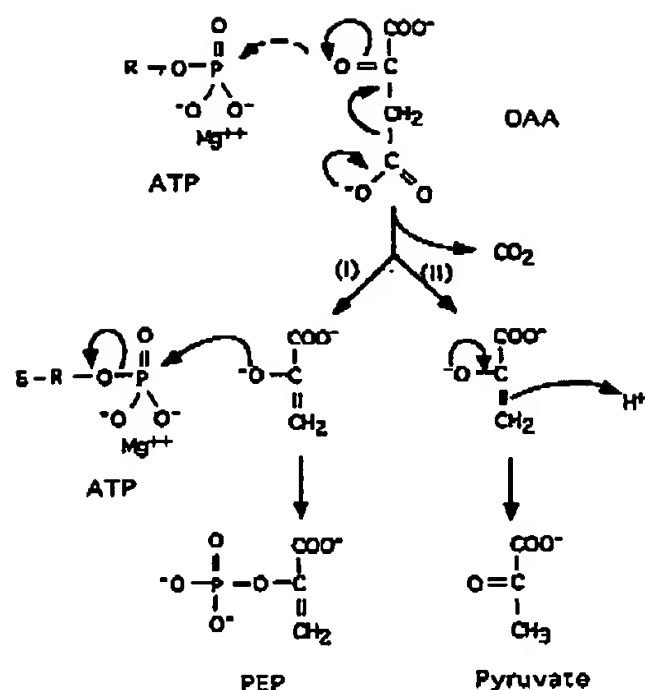
by the deduced protein sequence, in which the mutation on Pck51 is very close to the consensus ATP-binding site. Moreover, the fact that Oad activity is stimulated by ATP also suggests a role of the nucleotide in stabilizing the transitional intermediate (Fig. 4). However, the detailed mechanism remains to be investigated.

It is interesting that the wild-type E. coli Pck does not exhibit Oad activity while yeast and chicken liver mitochondrial Pck do. Apparently the Oad activity plays no role in E. coli physiology. When growth occurs on C4 carbon sources such as succinate, the two malic enzymes provide pyruvate while Pck provides PEP. In fact, diverting part of OAA to pyruvate may reduce the growth rate on the C4 carbon source, because Pck activity was already limiting the growth rate in succinate medium (3). To our knowledge, no malic enzyme activity has been found in liver mitochondria (11). Therefore, the Oad activity from Pck may play an essential role in the catabolism of aspartate and asparagine. An Oad activity which is not related to Pck and which is coupled to a membrane-bound sodium pump was found in Salmonella typhimurium (20). This enzyme is involved in citrate fermentation and contains three subunits which are encoded by oadG, oadA, and oadB. To our knowledge, this enzyme has not been found in E. coli.

The two Pck mutants isolated in this study indicate that Pck-induced growth inhibition is a result of PEP carboxykinase activity. When PEP-forming activity is changed to pyruvate-forming activity, growth inhibition disappears. The detailed mechanisms of this effect and its physiological significance are currently under investigation.

REFERENCES

1. Cannata, J. J. B., and A. O. M. Stoppani. 1963. Phosphopyruvate carboxylase from baker's yeast: III. The mechanism of oxaloacetate decarboxylation. J. Biol. Chem. 238:1919–1927.
2. Chao, Y.-P., and J. C. Liao. 1993. Alteration of growth yield by overexpression of phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase in *Escherichia coli*. Appl. Environ. Microbiol. 59:4261–4265.
3. Chao, Y.-P., R. Patnaik, W. D. Roof, R. F. Young, and J. C. Liao. 1993. Control of gluconeogenic growth by *pps* and *pck* in *Escherichia coli*. J. Bacteriol. 175:6939–6944.
4. Degnen, G. E., and E. C. Cox. 1974. Conditional mutator gene in *Escherichia coli*: isolation, mapping, and effector studies. J. Bacteriol. 117:477–487.
5. Fursta, J. P., W. Pansegrau, R. Frank, H. Blocker, P. Scholz, M. Bagdasarian, and E. Lanka. 1986. Molecular cloning of the plasmid RP4 primase region in a multi-host-range *incP* expression vector. Gene 48:119–131.
6. Goldie, A. H., and B. D. Sanwal. 1980. Genetic and physiological characterization of *Escherichia coli* mutants deficient in phosphoenolpyruvate carboxykinase activity. J. Bacteriol. 141:1115–1121.
7. Goldie, A. H., and B. D. Sanwal. 1980. Allosteric control by calcium and mechanism of desensitization of phosphoenolpyruvate carboxykinase of *Escherichia coli*. J. Biol. Chem. 255:1399–1405.
8. Goldie, H. 1984. Regulation of transcription of the *Escherichia coli* phosphoenolpyruvate carboxykinase locus: studies with *pck-lacZ* operon fusions. J. Bacteriol. 159:832–836.
8a. Goldie, H. Personal communication.
9. Goldie, H., and V. Medina. 1990. Physical and genetic analysis of the phosphoenolpyruvate carboxykinase (*pckA*) locus from *Escherichia coli* K12. Mol. Gen. Genet. 220:191–196.
10. Hansen, E. J., and E. Juni. 1975. Isolation of mutants of *Escherichia coli* lacking NAD- and NADP-linked malic enzyme activities. Biochem. Biophys. Res. Commun. 65:559–566.
11. Locher, G., A. A. Infante, I. Maurer-Fogy, E. Krystek, and M. B. Dworkin.

12. Medina, V., R. Pontarollo, D. Glaeske, H. Tabel, and H. Goldie. 1990. Sequence of the *pckA* gene of *Escherichia coli* K-12: relevance to genetic and allosteric regulation and homology of *E. coli* phosphoenolpyruvate carboxykinase with the enzymes from *Trypanosoma brucei* and *Saccharomyces cerevisiae*. J. Bacteriol. 172:7151–7156.
13. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
14. Noce, P. S., and M. F. Utter. 1975. Decarboxylation of oxaloacetate to pyruvate by purified avian liver phosphoenolpyruvate carboxykinase. J. Biol. Chem. 250:9099–9105.
14a. Osteras, M., T. M. Finan, and J. Stanley. 1991. Site-directed mutagenesis and DNA sequence of *pckA* of *Rhizobium* NGR234, encoding phosphoenolpyruvate carboxykinase: gluconeogenesis and host-dependent symbiotic phenotype. Mol. Gen. Genet. 230:257–269.
15. Parsons, M., and J. M. Smith. 1989. Trypanosome glycosomal protein P60 is homologous to phosphoenolpyruvate carboxykinase (ATP). Nucleic Acids Res. 17:6411.
16. Patnaik, R., and J. C. Liao. 1994. Engineering of *Escherichia coli* central metabolism for aromatic metabolite production with near theoretical yield. Appl. Environ. Microbiol. 60:3903–3908.
17. Stern, J. R. 1967. Oxalacetate decarboxylase of *Aerobacter aerogenes*. I. Inhibition by avidin and requirement for sodium ion. Biochemistry 6:3545–3551.
18. Stucka, R., M. D. Valdes-Hevia, C. Gancedo, C. Schwarzlose, and H. Feldmann. 1988. Nucleotide sequence of the phosphoenolpyruvate carboxykinase gene from *Saccharomyces cerevisiae*. Nucleic Acids Res. 16:10926.
19. Utter, M. F., and H. M. Kolenbrander. 1972. Formation of oxalacetate by CO_2 fixation on phosphoenolpyruvate, p. 117–168. *In* P. Boyer (ed.), The enzymes, vol. 6. Academic Press, New York.
20. Woehlke, G., K. Wifling, and P. Dimroth. 1992. Sequence of the sodium ion pump oxaloacetate decarboxylase from *Salmonella typhimurium*. J. Biol. Chem. 267:22798–22803.

1991. Human NAD$^+$-dependent mitochondrial malic enzyme: cDNA cloning, primary structure, and expression in *Escherichia coli*. J. Biol. Chem. 266:3016–3021.